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Total Mercury (THg), Lead (Pb), Cadmium (Cd) and Arsenic (As) in Hair Samples: Method Validation and Quantification among Women at Reproductive Age in Selangor

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Abstract

Mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As) have been known to cause toxicity to pregnant women, fetus and children. Method to detect these elements in human hair samples using single acid-microwave digestion-atomic absorption spectroscopy combination was validated and applied to quantify levels among women (n=311) at reproductive age. The value of $R^2 > 0.995$ for all four elements indicates an excellent and precise linear relation. Recovery between 90% - 110% along with RSD less than 10%, LOD between 0.1 – 0.3 ug/L and LOQ of hair samples between 0.09 – 0.24 µg/g describes a reliable efficiency to extract maximum toxicant level and quantify at moderately small level.

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Application of the validated method shows that 10.9%, 11.3%, 35.0% and 89.4% women had hair THg, Pb, Cd and As below the respective LOD. A significantly higher level of Hg ($p = 0.031$) and Pb ($p = 0.003$) was found in hair of coastal rural women. Also, 44.4% (95% CI = 38.5, 49.5) exceeded the EPA RfD of 1ug/g for hair Hg and a major portion of women (85.2%, 95% CI=81.3, 89.2) exceeded the WHO Pb maximum venerable level, 0.2ug/g. None of the respondents exceeded the WHO maximum venerable level of Cd, 10ug/g and the levels of as found were much lower than the ASTDR maximum level for non-exposed group, 1ug/g, except for five women.

Keywords: Human Hair; Total Mercury; Lead; Cadmium; Arsenic; Microwave acid digestion; Atomic absorption spectroscopy; Method Validation.

1. Introduction

Heavy metal contamination is a major environmental concern on global scale due exposure and intake by humans. The species and forms of a metal can define toxicity profile and target organ(s) [1], resulting in a range of toxicity including carcinogenicity, mutagenicity and teratogenicity. Mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As) have been studied numerously throughout the world and proven to cause adverse effects to mankind, especially during pregnancy [2– 4] due to high vulnerability of reproduction system and incomplete blood–brain barrier that affect developmental processes in fetus and young children upon maternal exposure [5]. Moreover, owing to differences as compared to the adult in many biochemical pathways, fetus are highly susceptible, typically at low exposure levels that do not harm the mother [6].

Toxicity monitoring is therefore vital, especially in the respective sensitive population. Human hair has been widely used in bio-monitoring of heavy metals in recent years to estimate environmental exposure levels and to assess nutritional status [7]. Studies found that hair has an unique ability to reflect the total body intake over an extended period of time in contrast to blood and urine which reflect the most recent exposure [8, 9]. Keratin that comprises in hair grows slowly allowing hair samples to be used as indicators for long-term exposure [10]. Furthermore segmental analysis of hair provides information about the time and duration of exposure, making it possible to map out changes over time depending on the length of the hair.

Numerous analytical methods have been developed and validated for elemental analysis in hair. Microwave-assisted digestion method offers time saving with reduce sample lost, eliminate exposure to corrosive acid fumes as compared to open digestions and prevent loss of volatile elements such as Hg or Pb [11]. Often, nitric acid (HNO_3)/hydrogen peroxide (H_2O_2) combination are chosen along with other mineral acids like hydrochloric acid (HCl), sulphuric acid (H_2SO_4) or phosphoric acid (H_3PO_4) [11–15]. Addition of H_2O_2 increases the oxidation potential of digestion whereby oxygen (O_2) evolved from the decomposition of H_2O_2 re-oxidises nitrous oxides (NOx), the reaction products between the organic samples to NO_3^- allowing HNO_3 to be "recycled" again [16]. However, the O_2 bubbles released required a long standing hours before microwave digestion can take place, thereby increasing the chances for sample/analyte lost especially the volatile Hg. On the other hand, in the presence of excess HNO_3, H_2O_2 had no beneficial property on the digestion [17].

Solubility of the resulting salts is a factor to be considered when deciding the suitable mineral acid to ensure the solutions remain stable for a longer period of time. While all nitrates salts are soluble, Pb forms insoluble salt with sulphates (SO_4^-) in H_2SO_4 whereas both Pb and Hg form insoluble salts with chloride (Cl^-) in HCl.

In order to prevent sample lost and formation of insoluble salts, nitric acid was chosen as the universal digester especially in detecting Pb and Hg. This paper aimed to validate a relatively simple microwave-nitric acid digestion and atomic absorption spectroscopy (AAS) technique to detect total Hg (THg), Pb, Cd and As in hair samples modified from [18]. The validation represents a tool which is used for proving the fact that a specific analytical method measures which pretends to measure is fitted for the desired purpose [19] and allow producing a reliable analytical data.

A certified reference material was used for this purpose. The levels in hair of urban and coastal rural women at child bearing age residing in Selangor, the most developed and dense state of Malaysia were then quantified using the same technique.

2. Methods

Analysis was conducted at the Laboratory of Vaccine and Immunotherapeutic (LIVES), Institute of Bioscience, University Putra Malaysia.

2.1. Reagents

Water used for sample preparation and cleaning of glassware in this study was ultrapure, 18.2 MΩ-cm (Elga PURELAB Ultra). All reagents were of analytical grade unless otherwise specified. Working standards of Hg Pb, Cd and As were procured from Perkin Elmer while Triton-X 100 laboratory grade from Sigma-Aldrich. All laboratory glassware and other utensils used in analyses were washed with a suitable detergent, soaked in 2M HNO_3 for at least 24 hours, rinsed in water and dried overnight in oven at 60ºC.

2.2. Reference Material

European human hair certified material (ERM DB001) was procured from European Commission, Joint Research Centre, Institute for Reference Material and Measurement, Belgium. ERMDB001 provide certification of the mass fraction of the total content of As, Cd, Cu, Hg, Pb, Se and Zn in human hair sample.

2.3. Instrumentation

Samples were digested in a microwave reduction system; Multiwave 3000, Rotor 16HF100 (100 ml PFA vessels, 40 bar) and p/T sensor accessory from Anton Paar. Elemental analysis were carried out using AAS: Hydride generation technique, VP90 Continuous Flow Vapour System (ThermoElemental VP90) with deuterium background correction was utilized for Hg [20–22]; GF 95 graphite furnace atomic absorption spectroscopy (GFAAS) with Zeeman background correction and FS95 furnace auto-sampler was employed to detect Pb, Cd and As.

2.4. Digestion procedure

About 0.1g of ERM DB001 samples were carefully measured into quartz digestive flask, 6.0ml of HNO_3 was pipetted in and the flasks were left for standing for 10minutes in fume cupboard. When no reactivity was observed, the flasks were sealed and samples were digested according to steps given in Table 1. After acid digestion, samples were cooled to room temperature and filtered through Whatman no. 1 filter papers into 50 mL volumetric flasks. Each solution was made up to 50 ml with water rinses of the residues and mixed thoroughly.

2.5. Quantification procedure

Quantification of the analyte was carried in VP90 Continuous Flow Vapour System for Hg according to analytical parameters given in Table 2 and G95-GFAAS for Pb, Cd and As according to analytical parameters given in Table 3. Working standards of Hg were prepared by diluting 1000ppm stock solutions of Hg with 25% v/v HCl whereas standards of Pb, Cd and As were prepared by diluting 1000mg/L of the respective stock solutions with 1% v/v HNO3. Linear equations were obtained by plotting peak area against concentration of standards at six calibration points in the range of 0 to 10 ug/L for Hg and 0 to 5 ug/L for Pb, Cd and As. Resulting samples peak area were replaced in the equation to obtain the corresponding concentration and converted further by adjusting with dilution factor and sample mass.

Table 1: Multiwave 3000 parameters for sample digestion

Parameter	Value	Parameter	Value		
			Temperature	Power	Time
Max Power Increase Rate	0.3 bar/s	Steps			
Max Pressure	50 kPa	1)Power ramp	-	1000 W	10 min
Max Microwave Power	1100 W	2)Power Hold	-	1000 W	20 min
IR Temp Limit	210˚C	3)Cooling	50˚C	0	10 min
Internal Temp Limit	280˚C				

Table 2: VP90 (D2 Quadline background correction) parameters for Hg quantification

Parameters	Value
Wavelength	253.7 nm
Carrier Gas	Nitrogen
Carrier Gas Flow Rate	50 ml/min
Reductant	0.6% m/V Sodium borohydride (NaBH4) solution stabilized in 0.6% m/V of Sodium hydroxide (NaOH)
Carrier Solution	HCl 25% v/v
Sample Volume	400 µl
Standby Delay	20 s
Stabilise Delay	50 s
Baseline Delay	40 s

Table 3: GF95 (Zeeman background correction) parameters for Pb, Cd & As quantification

Parameters	Pb	Cd	As
Wavelength (nm)	217	228.8	193.7
Bandwidth (nm)	0.5	0.5	0.5
Cuvette	ELC	ELC	ELC
Carrier gas	Argon	Argon	Argon
Gas flow	0.2 L/min	0.2 L/min	0.2 L/min
Modifier	$Mg(NO_3)_2$ 1% w/v	$Mg(NO_3)_2$ 1% w/v	$Ni(NO_3)_2$ 1% w/v
Working volume	20µl	20µl	20µl
Furnace Program	Temperature (˚C) / Time (s) / Ramp (˚C/s)		
Drying	100 / 30 / 10	130 / 25 / 10	140 / 15 /15
Ashing	800 / 20 / 150	300 / 10 / 5	600 / 10, 1.5 / 10
Atomising	1200 / 3 / 0	1800 / 1 / 0	2300 / 1 / 0.9
Cleaning	2500 / 3 / 0	2500 / 2 / 0	2600 / 3 / 1

2.6. Method Validation

Sample preparation was thoroughly validated to ensure credibility of the data in quantitative analyses. Factors considered were the determination coefficient (R^2) and linearity, recovery, precision, limit of detection (LOD) and limit of quantification (LOQ). A R^2 for linearity greater than 0.995 for each calibration curve was accepted [15]. The recovery of Hg, Pb and Cd was checked using the levels reported in ERM DB001 while recovery for As was checked by spiking ERM DB001 with As standard solution due to low level in ERM DB001 (0.045ug/g). Precision was measured from replicates of ERM DB001 samples, measured six times under repeatability conditions and six times at two different days under reproducibility conditions [23]. Based on guideline by association of analytical communities (AOAC), LOD was calculated as mean blank reading plus three times the standard deviation of blank and LOQ as mean blank reading plus 10 times the standard deviation of the blank [24]. Concentrations below detection limit were then replaced for convenience by half of sample LOD [13].

2.7. Application of Method

A total of 311 hair samples were collected from urban (n=164) and coastal rural (n=147) women of Selangor (Figure 1). A small lock of hair samples was tied with a cotton string at the occipital area and were cut 1cm from scalp into a clean polyethylene zipper plastic bag using a blunt-tipped stainless steel scissor [5, 25]. Scissors were cleaned prior to sampling with alcohol-free cleansing wipe. The samples were labelled, transported to lab and stored at cool, dark place until further analysis [26, 27]. Around 6-7 cm of hair samples were measured carefully using a pre-measured cotton string, cut to approximately 2-3 mm and transferred into a 15ml Pyrex tube with a screw cap. Samples were then washed three times with 1% v/v Triton-X, rinsed three times with water, and dried in an electric oven at 60 °C overnight [25, 26]. Samples were then digested and quantified using the same method as described for ERM DB 001.

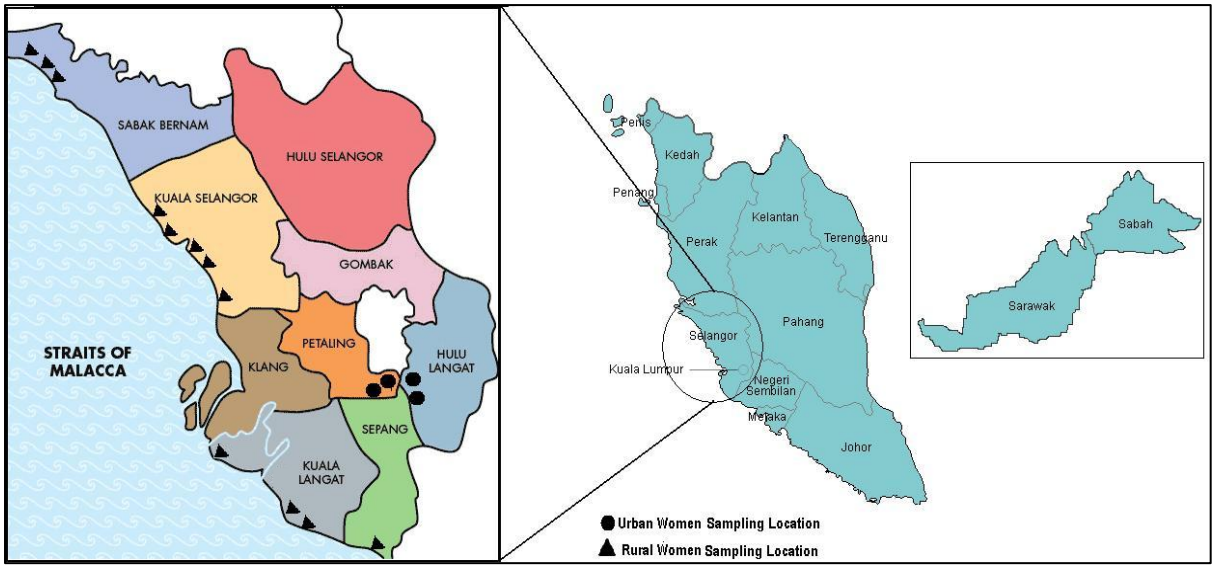

Figure 1: Location map of data collection

2.8. Quality control

Sample blanks were prepared for every digestion cycle to correct sample readings for any background or contamination in reagents, filters or distilled water used. Calibration check standard solutions were analyzed at the beginning, after every 10th sample and at the end of analysis, to monitor and control responses of the atomic absorption spectrometer [28].

2.9. Statistics

All statistical analyses were performed using IBM SPSS version 21.0. Descriptive and non-parametric statistics (Mann-Whitney U Test) were used due to violation of normality.

2.10. Ethics

The study was approved by UPM ethical committee; Reference: UPM/TNCPI/RMC/1.4.18.2 (FPSK-JKEUPM) INTV/F2. Respondents were explained about the purpose of the current research and informed consent was obtained prior to hair sample collection.

3. Results and Discussion

Procedures to determine the levels of THg, Pb, Cd and As in human hair samples using microwave acid digestion-AAS technique was validated to confirm that the requirements for the intended use or application have been met. A R^2 value greater than 0.990 and typically exceeded 0.995 are required for accurate quantification as analytical response is linear over certain concentration ranges or the peak height has a similar trend to that of relevant peak areas [29]. The R^2 value greater than 0.995 for all four elements in our study indicates an excellent and precise linear relationship between concentration and the corresponding peak area.

Method performance is demonstrated by acceptable recovery or accuracy; the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found [30]. Precision on the other hand expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions and is usually described as relative standard deviation (RSD). Recoveries for a sample in the range of 70-120% with a RSD ≤ 20% was accepted [11, 15, 26, 31–34]. In the present study, recoveries between 90% - 110% and RSDs < 10% (Table 4) show that the method able to perform maximum extraction repeatedly and for this reason the method was considered as ‘‘fit for purpose’’.

Performance of an instrument or an analysis is described by LOD and LOQ. The intent of LOD and LOQ is to define the smallest concentration of analyte that can be detected with no guarantee about the bias or imprecision of the result, the concentration at which quantitation as defined by bias and precision goals is feasible, and finally the concentration at which the analyte can be quantitated with a linear response [24]. The LOD and LOQ for hair samples were calculated for each elements by multiplying each LOD and LOQ by a factor of 500 (0.1 g sample and a final volume of 50 mL) [35]. Similar / closer detection limits for human samples analysis were found by [6, 36–40] for THg, [41, 42] for Pb, [25, 37, 38, 40,43] for Cd and [44, 45] for As . These shows, the validated method is applicable for hair sample elemental analysis using single acid, microwave acid digestion technique and AAS.

Table 4: Method Validation Estimates

	Mercury (Hg)	Lead (Pb)	Cadmium (Cd)	Arsenic (As)
LOD solution (ug/L)	0.23	0.27	0.15	0.26
LOQ solution (ug/L)	0.38	0.48	0.18	0.49
LOD hair samples (ug/g)	0.11	0.14	0.07	0.13
LOQ hair samples (µg/g)	0.19	0.24	0.09	0.25
Linear Range (ug/L)	10	5	5	5
Linear equation[a]	y = 0.00572x+0.0019	y = 0.02192x+0.0050	y = 0.08768x+0.0198	y = 0.00300x + 0.0044
R^2 ± SD	0.999 ± 0.001	0.999 ± 0.001	0.997 ± 0.002	0.998 ± 0.001
Precision (% RSD)				
- Repeatable	4.7	5.0	5.6	8.1
- Reproducible	8.1	6.9	8.5	8.8
Recovery[b]	96.35 ± 4.79	93.88 ± 3,23	103.36 ± 5.83	105.11 ± 6.23 [c]

[a] Linear equation for the best fit line

[b] Based on mean recovery of intraday assessment

[c] ERM DB001 spiked

Rapid growth in Malaysia has led to numerous anthropogenic activities that contributed to contamination and thus human exposure. The validated method was used to detect THg, Pb, Cd and As in hair samples collected from female respondents at reproductive age in urban and coastal rural part of Selangor. The results are given in Table 5. There were 10.9%, 11.3%, 35.0% and 89.4% women had hair THg, Pb, Cd and As below the LOD respectively. There were more urban women with no detectable levels of all the elements as compared to rural. Range shows that the lowest detected value was slightly higher than the calculated LOD. The median values were higher among rural women for THg, Pb and Cd. Nevertheless, Mann Whitney-U test gives a significant different between strata for THg and Pb with mean ranks higher for rural women. These show that coastal rural women are more exposed to THg and Pb than urban women. On the other hand, both coastal rural and urban women exhibit low levels of Cd and As which do not differ significantly across strata.

Hair THg, Pb, Cd and As found in the present study were compared with studies from other part of Malaysia and world (Table 6). Hair THg among rural women in the present study is comparable to the levels among rural female of Yan, Kedah whilst the urban women in this study had levels lower than that of Alor Setar, Kedah [46] which shows urban women of Selangor is less exposed. Hair Pb, Cd and As levels found are comparable to findings by [47]. Maximum level of Pb in the present study is comparable to 7.17 ug/g found by [48]. Also, As level is much lower than the level found in Penang (1.16 ug/g) [49], Kuala Lumpur (0.83 ug/g) and Sepang (0.27 ug/g) and Alor Setar (0.29 ug/g) by [50].

Table 5: Levels of THg, Pb, Cd and As based on strata; n=311(urban = 164, coastal rural = 147)

	Total below LOD (%)	Median (IQR) (µg/g)	Range[a] (µg/g)	Mean Rank[b]	p-value[c]
Mercury (Hg)					
Coastal Rural	8.2	0.98 (0.78)	0.29 – 5.40	167.63	0.031
Urban	13.4	0.82 (0.91)	0.13 – 4.98	145.58	
Total	10.9	0.91 (0.86)	0.13 – 5.40		
Lead (Pb)					
Coastal Rural	8.2	1.4 (1.57)	0.19 – 8.28	172.19	0.003
Urban	14.0	0.90 (1.27)	0.22 – 5.05	141.48	
Total	11.3	1.14 (1.46)	0.19 – 8.28		
Cadmium (Cd)					
Coastal Rural	34.7	0.20 (0.15)	0.10 – 1.39	151.14	0.366
Urban	35.4	0.21 (0.17)	0.10 – 1.89	160.36	
Total	35.0	0.20 (17)	0.10 – 1.89		
Arsenic (As)					
Coastal Rural	85.7	0.11 (0.00)	0.14 – 1.45	159.99	0.207
Urban	92.7	0.11 (0.00)	0.29 – 4.24	152.42	
Total	89.4	0.11 (0.00)	0.14 – 4.24		

[a] Range before replacing not detectable value with LOD/2

[b] Mann Whitney-U Test

[c] Significant value, $p<0.05$

Table 6: Concentration of THg, Pb, Cd and As in hair found in other studies

Element	Location	Population	Median	Mean	Range	Reference
Hg	South West Spain	Children	0.91	0.41	0.91	[42]
	Kedah (Yan)	Rural-Women	-	0.98	-	[46]
	Kedah (Alor Setar)	Urban-Women		1.16		
	Kelantan (Bachok)	Rural-Women	-	1.50	-	
	Kelantan (K.Bharu)	Urban-Women		1.14		
	Johor	Urban	-	9.84	0.60–19.76	[26]
		Rural		10.31	3.80–17.40	
	Terengganu	Urban	-	9.82	0.98–19.90	
		Rural		12.47	0.10–19.75	
	Kedah	Urban	-	11.41	0.05–20.50	
		Rural		15.99	3.36–21.00	
	Selangor	Urban	-	5.34	0.02–17.29	
		Rural		8.22	0.38–19.74	
	Cambodia-Phnom Penh	Urban	2.3	3.5	0.69 -190.00	[27]
	Cambodia-Kien Svay	Farming	2.6	3.2	0.54 – 70.00	
	Cambodia- Tomnup Rolork	Fishing	2.2	2.3	1.5 – 3.8	
	Cambodia- Batrong	Farming	2.9	2.8	1.1 – 7.5	
	Kuala Lumpur	Urban	3.38	4.01	0.59–18.37	[51]
	Jordan (Amman)	Urban	0.69	-	0.32 – 4.00	
	Libya (Beghazi)	Urban	0.81	-	0–3.60	
Pb	South West Spain	Children	<0.0913	<0.0913	-	[42]
	Israel (Nigev)	Urban	-	1.44	0.02-7.00	[47]
	Pakistan (Lahore)	Urban	-	3.53	-	[12]
Cd	South West Spain	Children	<0.0033	<0.0033	-	[42]
	Israel (Nigev)	Urban	0.10	-	<0.01 – 0.49	[47]
	Pakistan (Lahore)	Urban	-	0.08	-	[12]
As	South West Spain	Children	0.02	0.02	-	[42]
	Israel (Nigev)	Urban	0.01	-	<0.01 – 0.05	[47]
	Pakistan (Lahore)	Urban	-	0.31	-	[12]

Levels of THg, Pb, Cd and As in hair from the present study were also compared with international standards (Figure 2). The WHO guideline for maximum venerable level of Cd and Pb in hair/nail is 10ug/g and 0.2ug/g respectively [52] while Agency for Toxic Substances and Disease Registry (ASTDR) give 1ppm as the maximum level of arsenic in unexposed individuals [53].

From Figure 3, while none exceeded the Cd (Figure 2a) venerable level, the levels of As (Figure 2b) found were much lower than the ASTDR maximum level for unexposed group, except for five women, indicating less exposure for both Cd and As. A major portion of women (89.1%, 95% CI=85.1, 92.1) exceeded Pb maximum venerable level (Figure 2c) with 91.8% rural and 86.6% urban women.

As of hair THg, WHO (1990) reported no health effects for hair T-Hg below 50 µg/g based on neurotoxicity data from Japan and Iraq while United States Environmental Protection Agency (USEPA) adopted a revised reference dose (RfD) for MeHg of 0.1 µg Hg/kg body wt/day day based on neurological developmental effects measured in children associated with exposure in utero to MeHg from maternal diet and this was related to a hair THg concentration of 1.0µg/g [21]. On the other hand, Hg related neuropsychological dysfunctions were present in children with maternal hair Hg levels below 10 µg/g dry wt in the Tapajos River basin, Brazil and in the Faroe Islands, Denmark [54, 55].

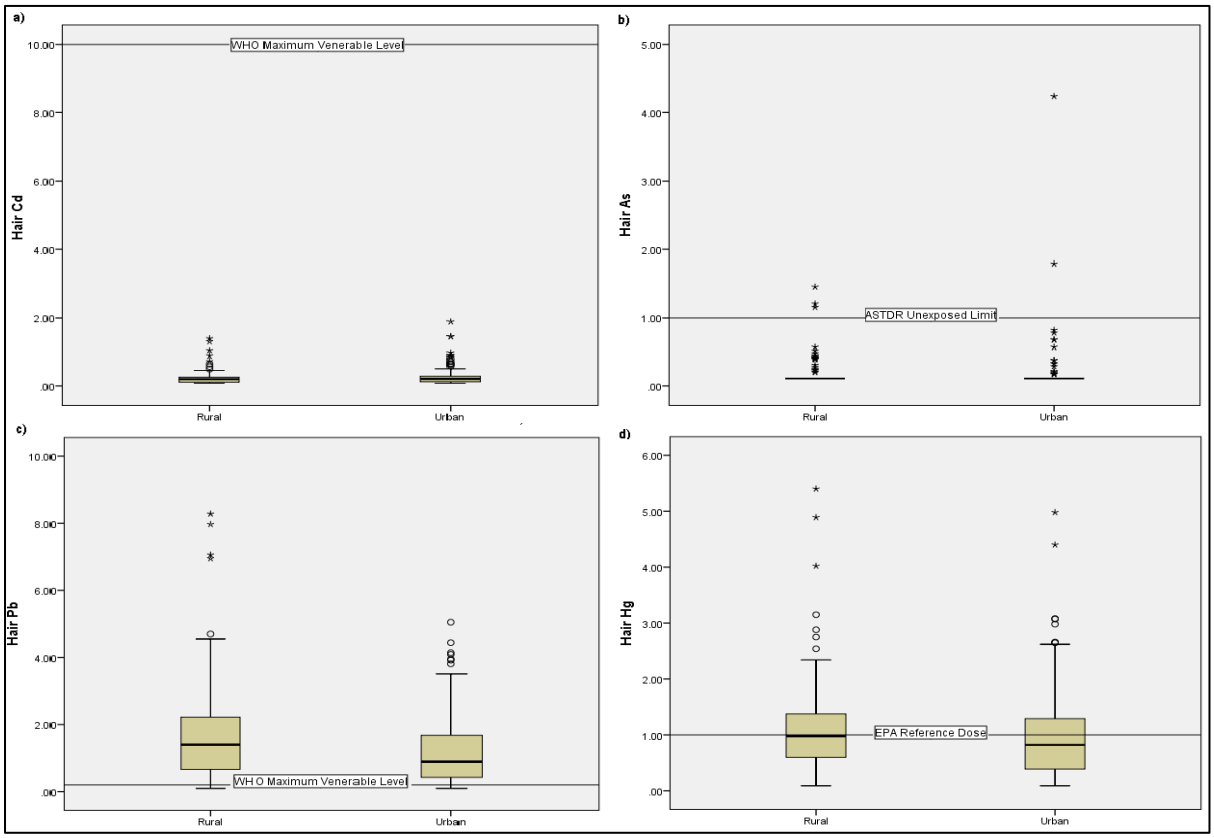

Figure 3: Distribution of THg, Pb, Cd and As in hair compared to international standards

In the present study, the respondents exceeded neither the no observable adverse effect level (NOAEL) for fetus neurotoxicity; 10µg/g nor the WHO neurotoxicity level; 50µg/g, similar to studies by [26, 46]. The levels found were also much lower than communities from Phnom Penh (city), Kien Svay (farming village), Tomnup Rolork (fishing village), and Batrong (farming village) in Cambodia whose hair THg ranged from 0.54µg/g up to 190µg/g with 12% exceeded the NOAEL for fetus neurotoxicity and three exceeded WHO neurotoxicity level [27]. Nonetheless, 44.7% (95% CI = 39.3, 50.3) women of present study exceeded the EPA RfD.

Cross tabulation shows that 49.0% coastal rural women and 40.9% urban exceeded the dose (Figure 3d). Yet, the percentage is lower compared to findings by [26, 46, 51]. This is probably due to the fact that the previous researchers included both male and female as respondents unlike the present study and Hg in the hair of males were found generally to be higher [27] as they consume higher amount of fish, the major Hg exposure route to mankind.

4. Conclusion

Methods to detect THg, Pb, Cd and As were validated and it was found that there are exposure to Hg and Pb greater than recommended level among women. During pregnancy and childbirth/nursing, exposure may be transferred to fetus and possibly lead to neurotoxicity. Further evaluation on causational factor including dietary habit is indeed necessary to ascertain the exposure source and plan the future risk management strategy.

5. Limitations

Analysis was done using AAS due to lack of funding to use the more advanced and preferred inductively coupled plasma mass spectrometry (ICPMS).

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